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Purification and Characterization of the Major Surface Array Protein from the Avirulent *Bacillus anthracis* Delta Sterne-1

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Many prokaryotic organisms possess surface layer (S-layer) proteins that are components of the outermost cell envelope. With immunogold labeling, it was demonstrated that the protein extractable antigen 1 (EA1) was localized on the outer surface and specifically to cell wall fragments from *Bacillus anthracis* which retained the S layer. When grown in rich medium under aerobic conditions, the avirulent strain Delta Sterne-1 released large amounts of EA1 into the medium. This EA1 had no higher-order structure initially but formed two-dimensional crystals under defined conditions. The released EA1 was purified in aqueous buffers with a three-step procedure and found to have a mass of 95 kDa when subjected to denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). N-terminal sequence data revealed exact identity to the first eight residues of the S-layer protein from *B. thuringiensis* 4045. Gel permeation chromatography of the purified EA1 under nondenaturing conditions revealed a single peak corresponding to a mass of approximately 400 kDa, suggesting that a tetramer or dimer of dimers was the primary species in solution. SDS-PAGE of EA1 purified in the absence of protease inhibitors revealed specific proteolytic processing to an 80-kDa form, which immunoreacted with polyclonal anti-EA1 antibodies. This proteolytic cleavage of EA1 to 80 kDa was duplicated with purified EA1 and the protease trypsin or pronase.

The gram-positive organism *Bacillus anthracis* is the etiological agent of anthrax and the only member of the genus *Bacillus* capable of causing invasive disease in humans and other mammals. Fully virulent strains of *B. anthracis* harbor two endogenous plasmids, pXO1 (22, 33) and pXO2 (10, 34), which code for the major known virulence factors of this organism, a poly-D-glutamic acid capsule and two bipartite exotoxins. The genes for capsule synthesis are located on the 95-kb plasmid pXO2 (10, 19, 20), while the genes for toxin synthesis are located on the 184-kb plasmid pXO1 (24, 28, 29, 35).

It was discovered during the late 1800s and early 1900s that cultures of virulent *B. anthracis* could be attenuated by growth at 42 to 43°C. The attenuation observed with such Pasteur-type vaccine strains resulted from curing the cells of the plasmid pXO1 (22). Fully virulent $pXO2^+$ $pXO1^+$ strains were thus attenuated by conversion to the $pXO1^-$ $pXO2^+$ genotype. Other attenuated strains, such as the Sterne strain, spontaneously lost pXO2 while retaining pXO1. Culturing the Sterne strain at 42°C resulted in the loss of pXO1 and produced the avirulent $pXO1^-$ $pXO2^-$ strain referred to as Delta Sterne-1 (15).

Immunological analysis of animals vaccinated with the current veterinary attenuated live spore vaccine revealed that serum antibody strongly recognizes a vegetative cell-associated protein termed extractable antigen 1 (EA1) (6). Two *B. anthracis* vegetative cell-associated proteins, EA1 and EA2, were initially identified from these studies (6). These highly antigenic proteins are extractable from vegetative cells or isolated cell walls with sodium dodecyl sulfate (SDS) or guanidine-HCl (6); however, the function and localization of EA1 were not defined. Previous electron microscopic analysis of *B. anthracis* Sterne cells demonstrated the presence of a paracrystalline layer on the surface of the bacterium, which was initially thought to have P6 or hexagonal symmetry (9, 13), although a subsequent, more thorough analysis suggested a unique P1 symmetry (4).

Crystalline surface arrays, now referred to as S layers, are an almost universal feature of outer cell envelopes of gram-positive and gram-negative eubacterial species, which represent nearly all phylogenetic lines (see references 14, 21, 31, and 32 for reviews). S layers are usually composed of a single protein or glycoprotein, which is associated with the peptidoglycan-containing cell wall in gram-positive eubacteria. S-layer proteins are capable of entropy-driven self-assembly into two-dimensional crystalline lattices characterized by defined symmetry and pores of uniform size (14, 31). Suggested functions for these unique proteins include acting as protective coats, molecular sieves, or ion traps, promoting cell adhesion and surface recognition, and providing frameworks that determine and maintain cell shape or rigidity (14, 21, 31, 32).

Evidence presented here demonstrates that EA1 is the major S-layer protein in *B. anthracis* Delta Sterne-1. We also describe the purification of the S-layer protein in aqueous buffers and the amino acid composition, N-terminal sequence, and proteolytic processing of EA1 from the Delta Sterne-1 strain.

(A preliminary version of this work was presented previously [8].)

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *B. anthracis* $pXO2^-$ Sterne and $pXO1^-$ $pXO2^-$ Delta Sterne-1 strains used in this study were obtained from Bruce Ivins (Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases). The Delta Sterne-1 strain was originally isolated by subculturing the toxigenic Sterne strain at 42°C (15). *B. anthracis* strains were grown at 37°C for 16 to 18 h on medium consisting of, per liter, 33 g of tryptone, 20 g of yeast extract, 2 g of L-histidine, 8 g of Na_2HPO_4, 7.4 g of NaCl, 4 g of KH_2PO_4, and 15 g of Bacto Agar adjusted to pH 7.4 with NaOH. Liquid cultures were grown in the same medium minus the Bacto Agar in loosely capped nonbaffled Erlenmeyer flasks filled to 50% of the flask volume and incubated at 37°C with

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shaking at 150 rpm for 16 h. The optical density at 600 nm (OD_{600}) was read on 10- to 20-fold dilutions of the cultures before harvesting at a final OD_{600} of 2.5 to 3.5. The relationship between OD_{600} and dry cell weight was linear for all strains under the conditions described here.

EA1 purification. One-liter cultures were centrifuged (10,800 × *g*) in a Sorvall GS-3 rotor (DuPont NEN Research Products, Boston, Mass.) for 30 min at 4°C. The supernatant was sterile filtered through cellulose acetate 0.2-μm-pore-size filters to remove residual cells and debris. The culture supernatant pH was stabilized with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.0) before freezing at −70°C. Sepharose CL-4B (Pharmacia, Piscataway, N.J.) (7% settled volume of Sepharose CL-4B per volume of supernatant) was washed with 10 volumes of Milli-Q ultrapure H_2O (Millipore Corp., Marlborough, Mass.) and added to thawed supernatant at 4°C while stirring. Solid ammonium sulfate was added to 70% of saturation, and the Sepharose CL-4B was stirred on ice for a minimum of 1 h before collection on a sintered glass filter under vacuum. The Sepharose CL-4B was washed with 1.0 liter of a 70% saturated ammonium sulfate solution prepared in 5 mM K_2HPO_4-KH_2PO_4 (pH 6.8) until the effluent appeared colorless. The EA1 was eluted from the Sepharose CL-4B with two 50-ml and two 25-ml washes with 5 mM K_2HPO_4-KH_2PO_4 (pH 6.8). The final combined eluate containing EA1 was dialyzed overnight at 6°C against 27 to 30 volumes of 5 mM K_2HPO_4-KH_2PO_4 (pH 8.0)–2 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride (PMSF). The dialyzed protein was applied at 6°C to an anion-exchange Macro Prep 50 Q (Bio-Rad, Richmond, Calif.) column (1 by 22 cm) equilibrated with 10 mM K_2HPO_4-KH_2PO_4 (pH 8.0) with a peristaltic pump at a flow rate of 1 ml/min. The EA1 remained unbound under these conditions, while several protein and nonproteinaceous contaminants were bound. The EA1 was collected, pooled with a 1-column-volume 10 mM K_2HPO_4-KH_2PO_4 (pH 8.0) wash, and dialyzed against 30 volumes of 5 mM K_2HPO_4-KH_2PO_4–2 mM EDTA–0.1 mM PMSF (pH 6.8) at 6°C. The partially purified EA1 was loaded onto an HTP hydroxylapatite (Bio-Rad) column (2.5 by 17 cm), equilibrated in 5 mM K_2HPO_4-KH_2PO_4 (pH 6.8), at 6°C. Under these conditions, the EA1 bound. The column was washed with the equilibration buffer at a flow rate of 1 ml/min until remaining nonproteinaceous contaminants were eluted and a stable baseline at 206 nm was achieved. The EA1 was eluted as a single peak with a linear 3.3-column-volume gradient to 1 M K_2HPO_4-KH_2PO_4 (pH 5.7). The fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and those containing pure EA1 were pooled and dialyzed against 5 mM K_2HPO_4-KH_2PO_4–1 mM EDTA–0.1 mM PMSF (pH 8.0). The concentration of purified protein was determined by using the Bio-Rad protein assay reagent as described by the manufacturer. The concentration of EA1 in crude extracts was determined from a linear standard curve of band area versus concentration of EA1 generated by SDS-PAGE. Briefly, known amounts of purified EA1 within the linear response range of the assay were subjected to SDS-PAGE, stained with Coomassie brilliant blue, and scanned with a laser densitometer (Pharmacia). Appropriate dilutions of crude samples containing unknown amounts of EA1 were applied to the same gel, and the 95-kDa regions of the gel were scanned. The band areas for unknown samples within the standard curve linear range were used to determine the concentrations of EA1 in those samples.

Formation of two-dimensional crystals. Crude culture supernatant from Delta Sterne-1 cells containing EA1 was bound to Sepharose CL-4B, washed, and eluted as described above. The partially pure EA1 was then dialyzed against 10 volumes of 10 mM K_2HPO_4-KH_2PO_4–2 mM EDTA–0.1 mM PMSF (pH 8.0) at 6°C overnight. Analysis of the EA1 by electron microscopy after negative staining revealed no detectable crystalline sheets. The EA1 was passed through a 0.2-μm-pore-size cellulose acetate filter, transferred to sterile dialysis tubing, and finally dialyzed against 67 volumes of sterile filtered 5 mM sodium acetate–1 mM EDTA–0.1 mM PMSF (pH 5.0). A white precipitate was visible after dialysis overnight at 6°C. The precipitate was centrifuged at 12,000 × *g* for 30 min and resuspended in dialysis buffer. Three-microliter samples were air dried on carbon support film grids, negatively stained with 2% (wt/vol) phosphotungstic acid, and viewed in a JEOL 1200 EX electron microscope at 60 kV.

GPC. Gel permeation chromatography (GPC) was carried out with a Superose 6 (Pharmacia) column (1 by 30 cm) with an exclusion limit of 4×10^7 Da for globular proteins or a Superdex 200 (Pharmacia) column (1 by 30 cm) with a 10^6-Da exclusion limit for globular proteins. Both columns were equilibrated and used in 10 mM K_2HPO_4-KH_2PO_4–150 mM NaCl (pH 7.3). The chromatograms were developed at a flow rate of 0.3 ml/min with a model 600E high-pressure liquid chromatography system equipped with Millenium 2001 software and a WISP 712 autoinjector (Waters, Marlborough, Mass.).

Rabbit polyclonal anti-EA1 preparation. Three aliquots of purified EA1 corresponding to 100 μg each were mixed with equal volumes of 4% (wt/vol) SDS–12% (vol/vol) glycerol–50 mM Na_2CO_3–2% (vol/vol) β-mercaptoethanol–0.01% (wt/vol) bromphenol blue and heated at 95°C for 10 min. An equal volume of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, Mo.) was added to one aliquot, and the mixture was homogenized by using two syringes connected by a 23-gauge adaptor. The other two aliquots were frozen at −20°C. The antigen-adjuvant emulsion was injected into a New Zealand White male rabbit, 0.5 ml intramuscularly in both thighs and 0.2 ml subcutaneously in the back. Preimmune serum was collected from the rabbit prior to injection of the EA1. Two further immunizations were administered after 4 and 10 weeks with the remaining 100-μg aliquots mixed with Freund's incomplete adjuvant. Anti-EA1 serum was collected 2 and 4 weeks after the final injection.

Monoclonal antibodies were prepared as described previously (7).

Immunolabeling for electron microscopy. Delta Sterne-1 cells for transmission electron microscopy (TEM) immunogold labeling experiments were cultured as described above, harvested by centrifugation, washed with 10 mM K_2HPO_4-KH_2PO_4–150 mM NaCl (pH 7.3), and fixed for at least 24 h in an aldehyde fixative composed of 4% (vol/vol) paraformaldehyde, 0.5% (vol/vol) glutaraldehyde, 100 mM sodium cacodylate, 1 mM $CaCl_2$, and 16 mM glucose. The fixed cells were washed with 20 mM Tris-HCl–0.9% (wt/vol) NaCl (pH 8.2), partially dehydrated in ethanol, and embedded in LR white resin (Polysciences Inc., Warrington, Pa.). One-hundred-nanometer sections were cut and collected on 300-mesh copper grids (Electron Microscopy Sciences, Fort Washington, Pa.). Nonspecific immunostaining was prevented by floating the grids, section-bearing side down, for 20 min on 4% (vol/vol) normal goat serum in 20 mM Tris-HCl–0.9% (wt/vol) NaCl–0.1% (wt/vol) bovine serum albumin (BSA)–0.05% (vol/vol) Tween 20–0.5% (wt/vol) gelatin (pH 8.2). Immunostaining was carried out for 1 h with drops of a 1:200 dilution of monoclonal anti-EA1 in 20 mM Tris-HCl–0.9% (wt/vol) NaCl–0.1% (wt/vol) BSA–20 mM NaN_3 (pH 7.5). After three 5-min washes in the same buffer, grids were incubated for 1 h with goat anti-rabbit immunoglobulin G (IgG) conjugated to 10-nm colloidal gold (Amersham Corp., Arlington Heights, Ill.). The negative controls were treated identically with the exception that the primary antibody was omitted. Sections were post-stained with 3.5% (wt/vol) uranyl acetate–0.2% (wt/vol) lead citrate and viewed on a JEOL 1200 EX TEM at 60 kV.

Samples for immunolabeling of sonicated cell wall fragments were prepared from Delta Sterne-1 cells grown overnight on plates. Cells were removed from areas of confluent growth with a swab and resuspended in 5 mM K_2HPO_4-KH_2PO_4–150 mM NaCl (pH 7.3). The cell suspension was transferred to a sonication cup attachment for a Branson model 450 400-W sonicator equipped with a microtip (Branson Inc., Danbury, Conn.). The cells were sonicated for 30-s intervals, at a 40% duty cycle mode, with the sample cup submerged in an ice bath. Samples were removed after each interval and mixed with a twofold concentration of the paraformaldehyde-glutaraldehyde fix described above. Samples of 3 μl were air dried on carbon support film grids (Electron Microscopy Sciences). The specimen-bearing grids were washed three times by floating on drops of 20 mM Tris-HCl–0.5 M NaCl (pH 7.5) for 5 min. Immunostaining was carried out as described above.

Immunoblot analysis of SDS cell extracts, secreted polypeptides, and purified EA1 with rabbit polyclonal anti-EA1. Protein samples for immunoblot analysis were diluted with an equal volume of 4% (wt/vol) SDS–12% (vol/vol) glycerol–50 mM Na_2CO_3–2% (vol/vol) β-mercaptoethanol–0.01% (wt/vol) bromphenol blue (pH 10.0) and heated at 95°C for 15 min before application onto 12.5% Tris-Tricine polyacrylamide gels (Integrated Separation Systems, Natick, Mass.). Proteins were electrotransferred onto 0.2-μm-pore-size nitrocellulose (Pierce, Rockford, Ill.) with a 25 mM Tris-HCl–192 mM glycine (pH 8.5)–20% (vol/vol) methanol transfer buffer at 50 V for 4 h. The nitrocellulose was blocked with 5 mM K_2HPO_4-KH_2PO_4–150 mM NaCl–0.5% (wt/vol) gelatin–0.05% (vol/vol) Tween 20 (pH 7.4) at room temperature for 2 h. Rabbit polyclonal anti-EA1 at a dilution of 1:10,000 in 5 mM K_2HPO_4-KH_2PO_4–150 mM NaCl–0.05% (vol/vol) Tween 20 (pH 7.4) was added, and the mixture was incubated for 1 h. The nitrocellulose was washed with 5 mM K_2HPO_4-KH_2PO_4–150 mM NaCl–0.05% (vol/vol) Tween 20 (pH 7.4) before goat anti-rabbit IgG conjugated to horseradish peroxidase was added at a 1:5,000 dilution in 5 mM K_2HPO_4-KH_2PO_4–150 mM NaCl–0.05% (vol/vol) Tween 20 (pH 7.4). Immunoreactive proteins were visualized with 5 mM 4-chloro-1-naphthol–0.1% (vol/vol) hydrogen peroxide in 5 mM K_2HPO_4-KH_2PO_4 (pH 7.4)–150 mM NaCl–17% (vol/vol) methanol.

In vitro proteolysis of purified EA1. Purified EA1 or protective antigen (PA) was diluted 1:1 with 50 mM HEPES–2 mM $CaCl_2$–1 mM EDTA (pH 7.5) to a final concentration of 100 μg/ml. Pronase (1 mg/ml) and trypsin (1 mg/ml) stocks were prepared in 25 mM HEPES–1 mM $CaCl_2$–0.5 mM EDTA (pH 7.5) immediately before use. Either trypsin or pronase was added to a final concentration of 0.3 μg/μg of substrate. Digestion was carried out at room temperature for 30 min. Reactions were stopped by adding SDS buffer and immersing the samples into a 95°C water bath. Samples were analyzed by SDS-PAGE with 12.5% Tris-Tricine gels.

Amino acid analysis of purified secreted EA1. A total of 325 μg of purified EA1 was desalted on a PD10 column (Bio-Rad) equilibrated with 5 mM NaCl–50 μM K_2HPO_4-KH_2PO_4 (pH 7.5). The desalted EA1 was frozen, lyophilized, and resuspended in 6 N constantly boiling HCl containing 0.01% (vol/vol) thioglycolic acid. The EA1 was split into three equal aliquots, transferred into amino acid vacuum hydrolysis vessels (Pierce), degassed under vacuum, and purged with argon before heating to 110°C for 24, 48, or 72 h. After hydrolysis, samples were dried under vacuum and resuspended in sodium citrate amino acid analysis buffer (pH 2.2) containing 2.0% (vol/vol) thiodiglycol and 0.1% (vol/vol) phenol (Pierce). Analysis was carried out on a model 6300 amino acid analyzer (Beckman, Fullerton, Calif.) under standard conditions as described by the manufacturer.

N-terminal analysis of purified secreted EA1 and EA1 extracted from the cell surface with SDS. N-terminal analysis of the soluble purified EA1 was carried out with approximately 100 pmol of desalted purified EA1 on a model 470A protein sequencer (Applied Biosystems Inc., Foster City, Calif.). Phenylthiohy-

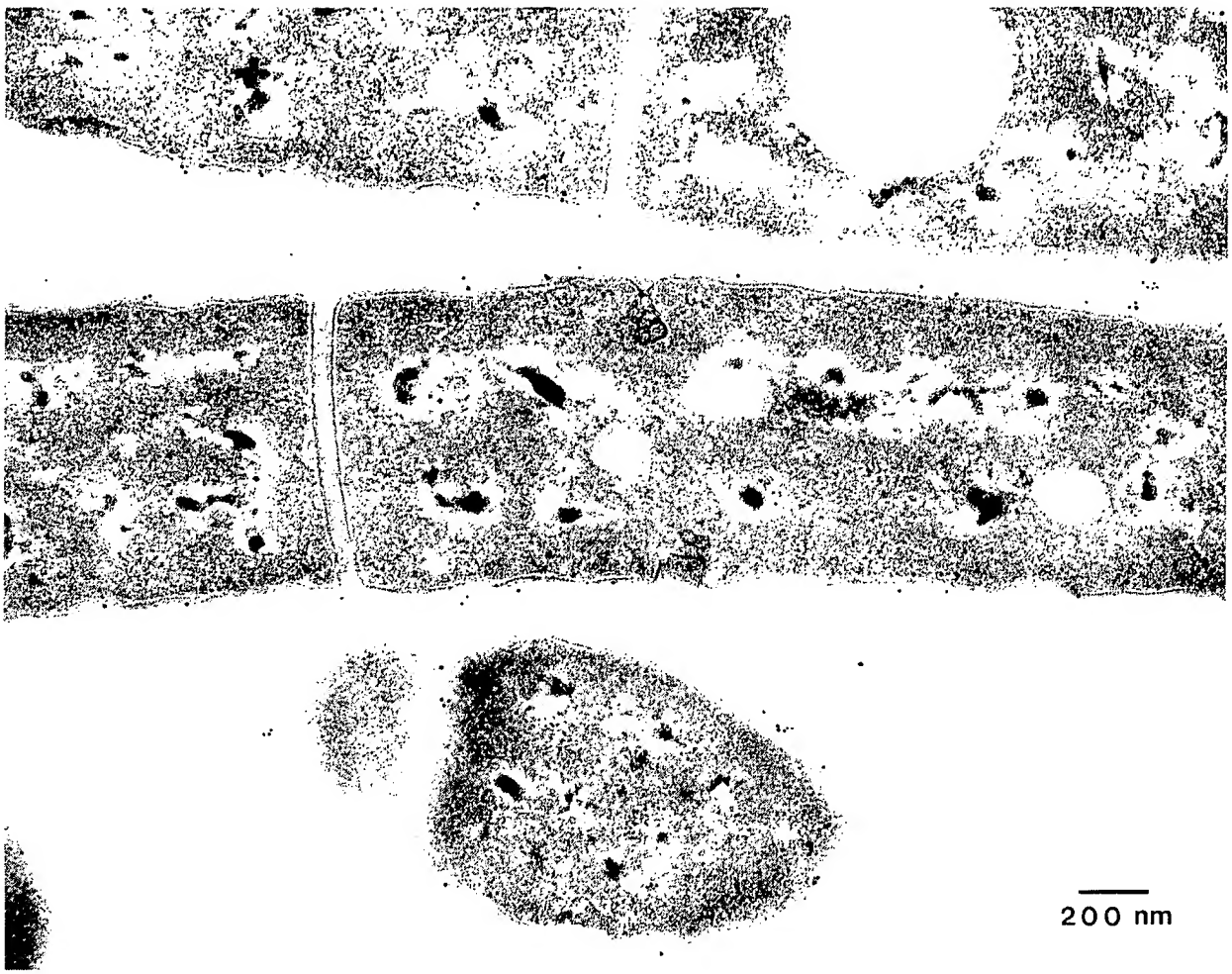

FIG. 1. Immunogold labeling of *B. anthracis* Delta Sterne-1 cells with mouse monoclonal anti-EA1 antibodies. Delta Sterne-1 cells were harvested from a stationary-phase culture, pelleted, washed, and fixed as described in Materials and Methods. The primary antibody was a cocktail of mouse anti-EA1 monoclonal antibodies EAII-4G10-1-1, EAII-7F9-1-2, and EAII-6C9-1-2 prepared against EA1 extracted from purified cell walls (6). Immunolabeling and TEM conditions were as described in Materials and Methods. Control experiments in which the primary antibody was omitted were unreactive.

dantoin-derived amino acids were identified with a model 120A phenylthiohydantoin analyzer from the same manufacturer. N-terminal analysis was also carried out on EA1 extracted from Delta Sterne-1 cells. Cells were washed twice with 50 mM Tris-HCl–50 mM NaCl–1 mM PMSF–1 mM EDTA–50 μM 1,10-phenanthroline (pH 8.0) and resuspended in the same buffer with 1% (wt/vol) SDS added. The cell suspension was briefly vortexed between two 10-min incubations on ice. Cells and large debris were removed by centrifugation, and the remaining protein was passed through a 0.22-μm-pore-size cellulose acetate filter. Samples were diluted 1:1 in SDS buffer, heated to 95°C, and applied to 12.5% Tris-Tricine gels. After electrophoresis was complete, the proteins were electrotransferred in 10 mM [3-(cyclohexylamino-1-propanesulfonic acid] (CAPS) (pH 11.0)–10% (vol/vol) methanol to Immobilon-P polyvinylidene difluoride membrane (Millipore) at 50 V for 30 min. The membrane was stained with 0.1% (wt/vol) Coomassie brilliant blue R-250 in 1% (vol/vol) acetic acid–40% (vol/vol) methanol and destained in 50% (vol/vol) methanol before the EA1 band was excised and sequenced.

Materials. Trypsin and pronase were obtained from Sigma, and affinity-purified, goat anti-rabbit IgG-horseradish peroxidase conjugate was purchased from Bio-Rad. Low-protein-binding 0.2-μm-pore-size cellulose acetate filters were from Millipore. Ultrapure-grade dialysis tubing for recrystallization experiments was obtained from Gibco (Gaithersburg, Md.). Tryptone, yeast extract, and Bacto Agar were from Difco (Detroit, Mich.). All other reagents and chemicals were obtained from Sigma, Aldrich, or Fisher Chemicals.

RESULTS

Immunolabeling of the Delta Sterne-1 cell surface with anti-EA1 monoclonal antibodies. In this study, monoclonal antibodies generated against EA1 purified from the cell wall were used with immunogold labeling to determine the localization of EA1 on *B. anthracis* Delta Sterne-1 cells. Figure 1 shows the immunogold labeling of thin-sectioned cells with a mixture composed of three monoclonal antibodies and anti-mouse IgG conjugated to 10-nm gold particles. The cells for this study were washed extensively to remove cellular debris, including proteins released from the cells into the culture supernatant. The specific labeling confirmed the earlier suggestion that EA1 was associated with the vegetative cell and localized the EA1 to the cell surface. Immunolabeling of washed Delta Sterne-1 cells with polyclonal antibodies directed against EA1 purified from culture supernatant and anti-rabbit IgG conjugated to 10-nm gold particles also revealed specific staining of the cell surface (data not shown).

The surface localization of immunoreactive EA1 and the

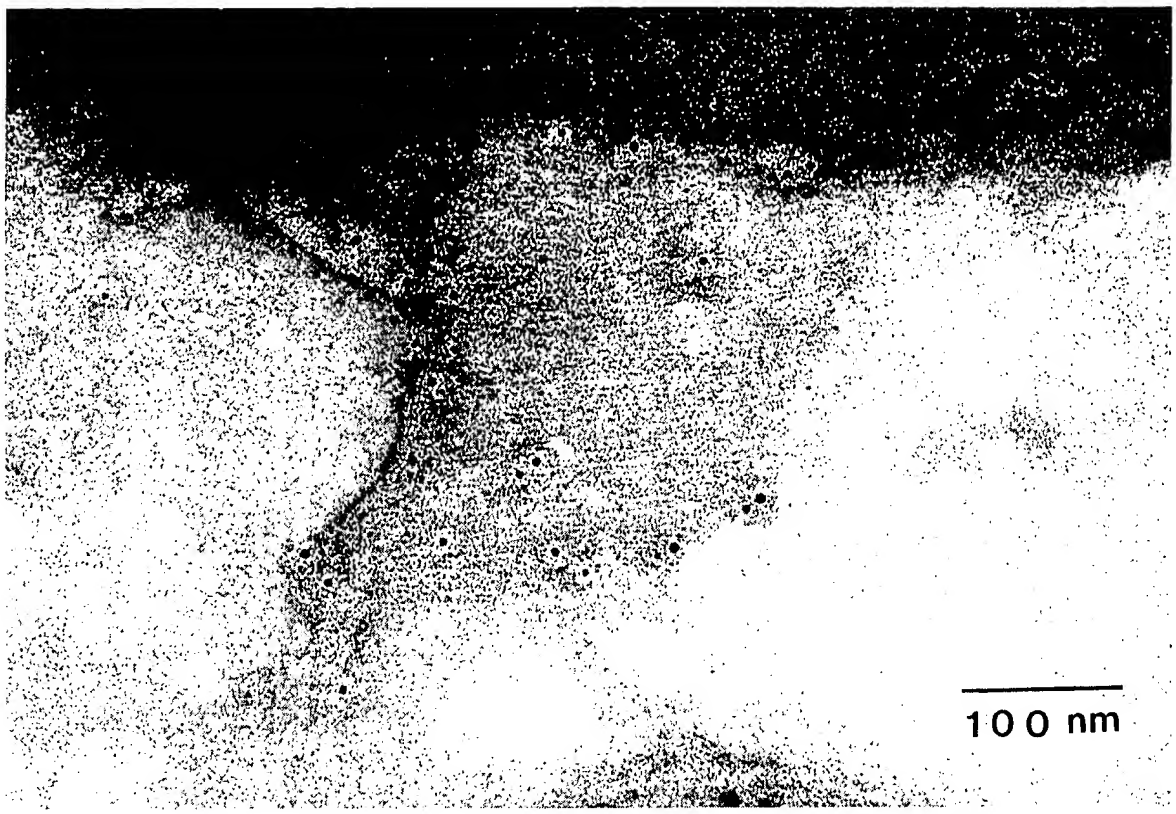

FIG. 2. Immunolabeling of cell wall fragments containing regular arrayed protein dislodged from sonicated Delta Sterne-1 cells. Delta Sterne-1 cells were grown, washed, and sonicated as described in Materials and Methods. Immunolabeling was carried out as described in Materials and Methods, using a 1:200 dilution of rabbit polyclonal anti-EA1 prepared against purified EA1.

similarity in size between the 95-kDa EA1 and the 91.5-kDa S-layer protein from the closely related species *Bacillus thuringiensis* 4045 (18) suggested that EA1 might function as an S-layer protein in *B. anthracis*. To test this possibility further, *B. anthracis* Delta Sterne-1 cells were cultured in rich medium and disrupted by sonication, and cell wall fragments were harvested. Electron microscopic analysis after negative staining revealed that some of these cell wall fragments still contained bound sheets of regular arrays of subunits characteristic of a bacterial crystalline S layer (4, 9, 13). The S layer attached to the cell wall fragments was immunoreactive when probed with polyclonal anti-EA1 antibodies (Fig. 2). This finding provided the first direct evidence that the S layer contains EA1 as a major component.

Characterization and comparison of released and cell-associated EA1 produced from attenuated *B. anthracis* Sterne and avirulent Delta Sterne-1. When Sterne and Delta Sterne-1 were cultured in R medium, a minimal defined medium (27), both strains produced EA1 that could be extracted from the cell surface by SDS or guanidine-HCl but did not release detectable levels into the culture supernatant (7a). However, when grown in the rich medium, both strains released EA1 into the culture supernatant. Assay of EA1 released by the two strains under identical growth conditions demonstrated that the levels of EA1 released for both strains were independent of the growth phase or aeration levels. The quantitative analysis of released EA1 by SDS-PAGE revealed that the amount of EA1 released by Delta Sterne-1 was more than twofold higher

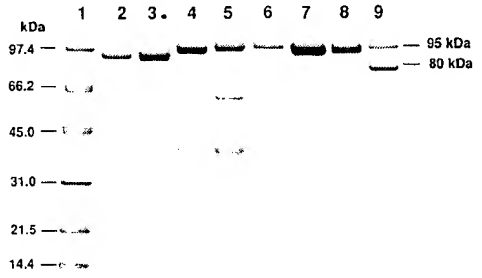

FIG. 3. SDS-PAGE of SDS cell extracts and culture supernatants from Delta Sterne-1 and Sterne cells. All cells were cultured in rich medium to the same final OD_{600}. The linear relationship between dry weight and OD_{600} was confirmed for both strains throughout the growth curve to rule out differences in cell mass sampled. Equivalent volumes of supernatants or SDS cell extracts were concentrated in Centricon 30 concentrators to equal final volumes, solubilized in equal volumes of SDS solubilization buffer, and heated for 5 min at 95°C before 10 μl of each sample was subjected to SDS-PAGE. Lanes: 1, low-molecular-weight-range SDS-PAGE protein standards (Bio-Rad); 2, *B. anthracis* PA, 1 μg; 3, *B. anthracis* lethal factor, 1 μg; lane 4, 1% (wt/vol) SDS extract of washed Sterne cells; 5, 1% (wt/vol) SDS extract of washed Delta Sterne-1 cells; 6, supernatant of Sterne culture; 7, supernatant of Delta Sterne-1 culture; 8, EA1 purified from supernatant; 9, EA1 purified from supernatant in the absence of protease inhibitors.

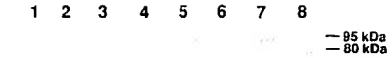

FIG. 4. Immunoblot analysis of SDS cell extracts, secreted polypeptides, and purified EA1 with rabbit polyclonal anti-EA1. Samples were identical to those used for Fig. 3. Lanes: 1, *B. anthracis* PA, 1 μg; 2, *B. anthracis* lethal factor, 1 μg; 3, 1% (wt/vol) SDS extract of washed Sterne cells; 4, 1% (wt/vol) SDS extract of washed Delta Sterne-1 cells; 5, supernatant of Sterne culture; 6, supernatant of Delta Sterne-1 culture; 7, EA1 purified from supernatant; 8, EA1 purified from supernatant in the absence of protease inhibitors.

than that released by the Sterne strain. These levels of EA1 were observed even after the strains were serially subcultured five times. This observed stability of the EA1 levels released into the medium was in contrast to the reported instability of the S-layer protein from *B. thuringiensis* 4045, in which case complete loss of expression was observed after three consecutive serial subculturings (18).

Figure 3 shows SDS-PAGE analysis of the SDS extracts of Sterne and Delta Sterne-1 vegetative cells (lanes 4 and 5, respectively). Lanes 6 and 7 contain concentrates of the culture supernatants from the same cultures. The predominant protein in each case was a 95-kDa species. An immunoblot of the same samples developed with polyclonal anti-EA1 (Fig. 4) revealed that the 95-kDa proteins from cell extracts (lanes 3 and 4) and culture supernatants (lanes 5 and 6) were both immunoreactive. On the basis of the similar electrophoretic mobilities and immunoreactivities with anti-EA1 antibodies generated against EA1 isolated from the supernatants, it was concluded that the 95-kDa protein product obtained from the culture supernatants was indistinguishable from that extracted from the cell surface of either strain by using SDS. The minor differences in the electrophoretic mobilities of the 95-kDa EA1 seen in Fig. 3 were attributed to differences in the total protein loaded per lane. The secreted 83-kDa *B. anthracis* PA and 86-kDa lethal factor were included in Fig. 3 as additional molecular weight standards and in Fig. 4 to demonstrate the specificity of the immunoreaction.

Macromolecular assembly of released EA1. The immunoreactivity of S-layer-containing cell wall fragments with anti-EA1 and the surface localization of the EA1 strongly supported the concept that EA1 was a component of the *B. anthracis* surface array but did not demonstrate that EA1 was an S-layer protein. To examine this matter further, the functionality of released EA1 in macromolecular assembly was investigated. An exhaustive TEM analysis of EA1 recovered from the culture supernatant and partially purified by using Sepharose CL-4B and ammonium sulfate failed to reveal the presence of any crystalline arrays or other distinguishable structures. However, upon dialysis against sodium acetate buffer at pH 5.0, a visible precipitate formed that was highly enriched in EA1 when examined with SDS-PAGE. The presence of small sheets of crystalline arrays (Fig. 5) or extended cylindrical shapes (data not shown) was revealed with TEM after negative staining. Although the symmetry of these crystalline arrays was not determined, the observed center-to-center unit spacing of 8 to 10 nm was consistent with previous results determined for intact cells (9, 13).

Purification of EA1 from Delta Sterne-1 culture supernatant. Soluble EA1 was separated from the Delta Sterne-1 culture by centrifugation and filtration through 0.2-μm-pore-size low-protein-binding cellulose acetate filters. As a first step in the purification, EA1 was bound to Sepharose CL-4B and separated from several pigmented nonproteinaceous contaminants. The removal of the pigmented material was critical to the success of purification since these contaminants interfered with the adsorption of the protein in subsequent purification steps and absorbed strongly in the UV range, making detection of proteins difficult. Once these contaminants were removed, protease inhibitors were added and the purification was completed by using quaternary amine ion-exchange and hydroxyapatite columns to remove the remaining minor protein contaminants, which varied on the basis of culture age and final density. The combined increased phosphate and decreased pH conditions used to elute the EA1 from the hydroxyapatite column enhanced the resolution and yielded pure EA1 (Fig. 3, lane 8). Anthrone analysis (16) of purified EA1 in solution and periodic acid-Schiff staining (17) of purified EA1 after SDS-PAGE failed to reveal detectable levels of carbohydrate.

Immunoblot analysis of purified EA1 from Delta Sterne-1 (Fig. 4, lane 7) with polyclonal anti-EA1 revealed that the purified product was immunoreactive. Further immunoblotting carried out with several preparations of EA1 purified in the presence and absence of protease inhibitors EDTA and PMSF revealed that the 80-kDa protein, which copurified in the absence of protease inhibitors, was strongly immunoreactive. In subsequent experiments, it was determined that prolonged incubation at 4°C in the absence of protease inhibitors resulted in the concomitant decrease in the 95-kDa and increase in the 80-kDa immunoreactive species (Fig. 4, lane 8), suggesting that the 80-kDa form resulted from the proteolytic degradation of the 95-kDa EA1.

Characterization of the purified EA1. The amino acid analysis of purified EA1 described in Materials and Methods is summarized in Table 1. The results are averages of three time points, 24, 48, and 72 h. The analysis results for the three time points were quite stable and consistent with the absence of large amounts of carbohydrate in the purified sample. The content of 47% nonpolar residues was similar to the values 44 to 50% determined for other *Bacillus* S-layer proteins analyzed to date (25, 31). The contents of 15% hydroxyl and 15% basic residues were also within published ranges of 11 to 18% for hydroxyl and basic residues in *Bacillus* S-layer proteins (25, 31). The calculated value of 1.40 for the ratio of charged to hydrophobic amino acids (ratio 3 [2]) and discriminant function (3) of 0.123 for EA1 were consistent with a peripheral protein and also agreed well with the ratio 3 (1.1 to 1.5) and discriminant function values (0.13 to 0.25) determined for other *Bacillus* S-layer proteins (25, 31).

The N-terminal sequence was determined for the soluble, purified 95-kDa EA1 and for EA1 extracted from the cell surface with SDS. The sequences for the first 18 cycles from the purified EA1 and the first 10 cycles of the SDS-extracted EA1 are shown in Fig. 6. Clearly, the sequences for both samples line up exactly, with the only point of ambiguity being cycle 4 of the SDS-extracted sample. The reason for the ambiguity was not clear. A search of the literature revealed an identical match with residues 2 through 8 of the S-layer protein from *B. thuringiensis* 4045 (18).

Because the purified EA1 degraded to an 80-kDa form in the absence of protease inhibitors, experiments were conducted to determine if the purified 95-kDa EA1 could be

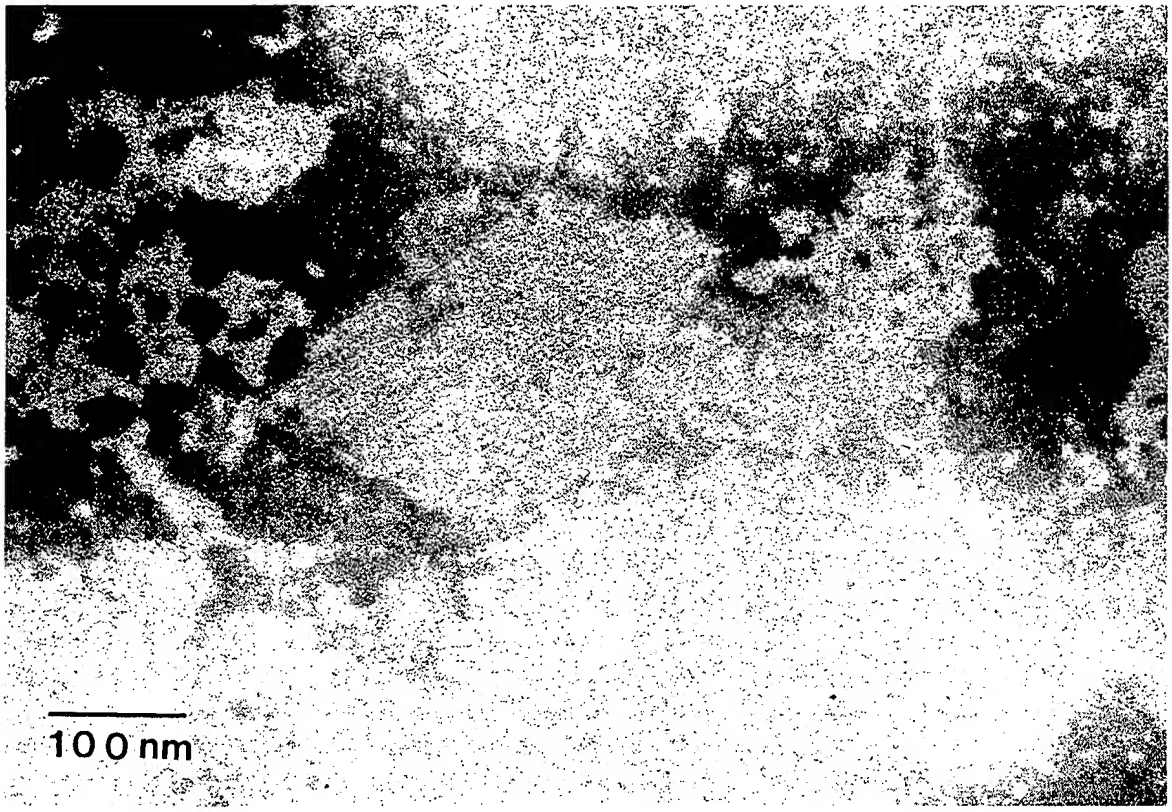

FIG. 5. TEM of negatively stained EA1 crystallized from partially purified EA1. The EA1 used for these experiments was partially purified by ammonium sulfate fractionation on Sepharose CL-4B. Samples were eluted from Sepharose as described in Materials and Methods. Samples were subjected to SDS-PAGE to confirm the isolation of EA1 and that proteolysis had not occurred. The crystallization conditions were described in Materials and Methods.

TABLE 1. Amino acid analysis of purified released EA1[a]

Amino acid residue	Mol%
Asx	10.3
Thr	8.3
Ser	4.8
Glx	13.2
Gly	7.2
Ala	13.1
Val	12.4
Met	0.9
Ile	2.8
Leu	6.9
Tyr	1.7
Phe	2.8
Lys	13.8
His	0.5
Arg	0.6
Pro	2.6
Cys	ND
Trp	ND

[a] Purified EA1 shown in Fig. 3 (lane 8) was desalted and hydrolyzed as described in Materials and Methods. Values represent averages of 24-, 48-, and 72-h hydrolysis time points unless otherwise noted. Values for serine and threonine were extrapolated to time zero; those for valine, leucine, and isoleucine were taken from the 72-h sample. Tryptophan and cysteine were not determined (ND). *S*-2-Aminoethyl-L-cysteine was used as an internal standard.

processed in this manner in vitro and to determine the protease specificity of the cleavage. Figure 7 shows that it was possible to partially process the EA1 to an 80-kDa form in vitro by using trypsin (lane 5) or pronase (lane 6). Proteolytic fragments with the same apparent M_r were also obtained with chymotrypsin (data not shown), which suggested that the processing did not require cleavage at a specific residue. The overall activity of the enzymes was confirmed by the obvious degradation of PA under the same conditions (lanes 2 and 3) to a 63-kDa form or lower-molecular-weight polypeptides.

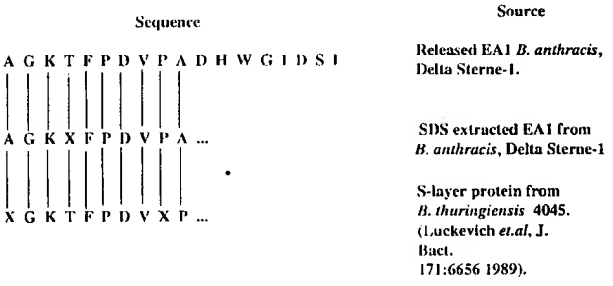

FIG. 6. N-terminal sequences of purified released EA1 and SDS-extracted EA1 and comparison with the N-terminal sequence of the S-layer protein from *B. thuringiensis* 4045. Purified EA1 shown in Fig. 3 (lane 8) was desalted as described in Materials and Methods before application to the sequenator. No secondary sequences were observed in either the SDS-extracted or purified samples.

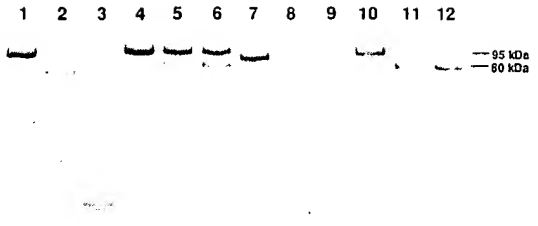

FIG. 7. In vitro proteolysis of purified EA1. Details of proteolysis are given in Materials and Methods. A total of 1 μg of each sample was loaded as follows: lane 1, PA; lane 2, PA plus trypsin; lane 3, PA plus pronase; lane 4, purified EA1; lane 5, purified EA1 plus trypsin; lane 6, purified EA1 plus pronase; lane 7, PA plus 2 M urea; lane 8, PA plus 2 M urea plus trypsin; lane 9, PA plus 2 M urea plus pronase; lane 10, purified EA1 plus 2 M urea; lane 11, purified EA1 plus 2 M urea plus trypsin; lane 12, purified EA1 plus 2 M urea plus pronase.

When the experiment was repeated in the presence of 2 M urea, the control was degraded to low-molecular-weight unresolved peptides, while EA1 was specifically and nearly quantitatively processed from 95 to 80 kDa (lanes 11 and 12).

As a final step in the characterization, the apparent molecular mass of the purified 95-kDa EA1 was determined in solution by GPC under nondenaturing conditions. When purified 95-kDa EA1 was applied to a Superose 6 column, it eluted as a single major symmetric peak (Fig. 8), corresponding to a molecular mass of approximately 400 kDa (Fig. 8, calibration curve inset). The single peak was consistent with the product being monodisperse and in the form of a tetramer or a dimer of dimers. The same behavior was also observed on a Superdex 200 GPC column (data not shown). Adding 2 M urea or guanidine-HCl to the buffer did not significantly alter the peak shape or elution volume. GPC of EA1 containing the 80-kDa proteolytically cleaved EA1 also eluted at the same volume but displayed a slight increase in peak asymmetry on the lower-molecular-weight side of the peak. The presence of the 80-kDa form in the same peak was confirmed by collecting multiple fractions across the peak and analyzing the protein content by SDS-PAGE (data not shown). Aside from the peak asymmetry, no other changes occurred in the chromatogram, suggesting that the 80-kDa species remained monodisperse in an associated form and was unresolved because of the minor change in molecular weight.

DISCUSSION

The specific immunoreactivity of polyclonal and monoclonal anti-EA1 antibodies with the cell surface of intact Delta Sterne-1 cells (Fig. 1) and cell wall fragments containing regular arrayed protein on the surface (Fig. 2) suggested that EA1 could be a *B. anthracis* S-layer protein. Further direct evidence was provided by the formation of sheets of crystalline arrays from partially purified EA1. These two-dimensional crystals displayed the same 8- to 10-nm center-to-center unit repeat distance as observed for the S layer on the cell surface (9, 13).

Identifying EA1 in the pXO1$^-$ pXO2$^-$ Delta Sterne-1 strain S layer made it apparent that EA1 was genomically encoded and that the endogenous plasmids were not required for expression or assembly of the S layer. A previous report identified EA2 as a second cell surface protein and suggested that it was coded for on the endogenous plasmid pXO1 (6). Because Delta Sterne-1 does not produce EA2, another vegetative cell-associated protein (6), the role of that protein remains unclear, but the data presented here suggest that it is not required for the production or assembly of the *B. anthracis* S layer under the conditions examined here.

Quantitative comparison of EA1 amounts released by

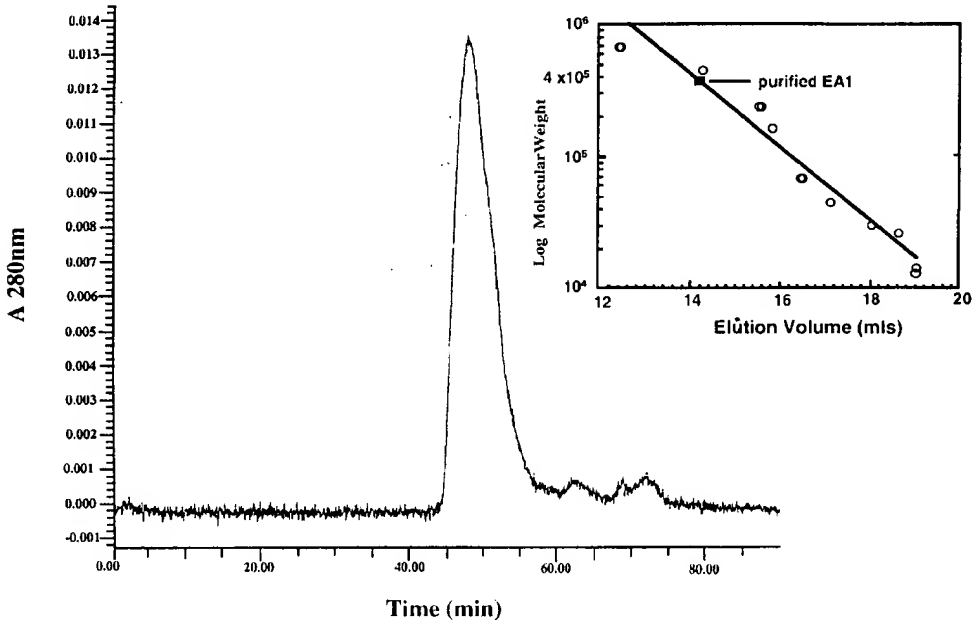

FIG. 8. Gel permeation analysis of purified EA1 under nondenaturing conditions. The gel permeation experiment was carried out with a Pharmacia Superose 6 column. All proteins were dissolved in 10 mM K_2HPO_4-KH_2PO_4–150 mM NaCl (pH 7.3), and injection volumes were held constant at 100 μl. Sample detection was at 280 nm. Standard proteins used for the calibration curve (inset) were thyroglobulin, ferritin, catalase, BSA, aldolase, ovalbumin, chymotrypsinogen, carbonic anhydrase, RNase, and cytochrome *c* (horse heart). The EA1 sample was shown in Fig. 3, lane 8.

Sterne and Delta Sterne-1 revealed a more than twofold-higher level of EA1 released by the latter. In the absence of data on total EA1 released, as well as assembled, at the cell surface, an unequivocal distinction cannot be made between increased EA1 production and decreased assembly at the cell surface. Although high levels of secretion have been observed with *Bacillus* species, most notably *B. brevis* (23), S-layer protein production is generally regarded as a tightly regulated process (1, 30). The increased EA1 production in the absence of pXO1 that we observed suggests an apparent direct or indirect *trans*-acting regulatory role for pXO1 in EA1 production, a phenomenon which has not been previously described.

Analysis of purified EA1 by GPC revealed a single molecular species of 400 kDa (Fig. 8). On the basis of recent reports of S-layer proteins being glycosylated (11, 21) and previous reports of strong association with isolated cell walls (12), carbohydrate analysis was carried out with the anthrone and periodic acid-Schiff assays. The absence of a detectable response suggested that the 400-kDa molecular species observed with GPC was determined primarily by protein, although low-level glycosylation cannot be rigorously excluded.

The monodisperse GPC behavior of EA1 described here indicated the isolation of a water-soluble minimal unit consisting of a tetramer or dimer of dimers. It is difficult at present to distinguish between the two possibilities, as the symmetry of the S layer in *B. anthracis* remains undetermined. Although EA1 initially was believed to have hexagonal symmetry (9, 13), a more recent and thorough investigation suggests that it may have a unique P1 symmetry (4). In the absence of more precise structural information on the native protein on the cell surface, the possibility also exists that EA1 has assumed a conformation or aggregate which, despite its competence for macromolecular assembly, is not physiologically significant in in vivo assembly.

Comparison of the N-terminal sequences of EA1 and other S-layer proteins revealed that seven of the first eight residues of the EA1 sequence were identical to the published sequence of the major S-layer protein from *B. thuringiensis* 4045 (18). Although the sequence identity was surprising, similarities between *B. anthracis* and *B. thuringiensis* had been observed previously. Studies of hyperimmune guinea pig antiserum against EA1 demonstrated a strong immunofluorescence response with 10 of 12 strains of unencapsulated *B. anthracis* and with several strains of *B. cereus* and *B. thuringiensis* (26). The cross-reactivity of anti-EA1 with other strains was pursued further with five monoclonal antibodies against EA1 (EA-II-7F9, EA-II-10D3, EA-II-6C9, EA-II-7B9, and EA-I-4G10). Several strains of *B. thuringiensis*, including ATCC 4040, 4041, and most notably 4045, cross-reacted strongly. In addition, *B. cereus* NRS 820 and ATCC 7064 also cross-reacted, while several other strains of *B. thuringiensis*, *B. cereus*, *B. alvei*, *B. coagulans*, *B. eugilitis*, *B. lentus*, *B. licheniformis*, *B. subtilis*, and *B. megaterium* did not. The cross-reactivity with the monoclonal antibodies and the similarities in M_r values on SDS-PAGE and in the N-terminal sequence between *B. anthracis* and *B. thuringiensis* 4045 suggest that the S-layer proteins from these species may be closely related or share common domains. The possibility of shared small domains has not been reported previously for different *Bacillus* species, although the three distinct S-layer proteins expressed in variants of *B. stearothermophilus* NRS 2004/3a under different growth conditions shared common sequence for the first five amino acids (30). As the monoclonal antibodies used here have not been mapped, the possibility that only the N-terminal portion of EA1 is conserved throughout these different species cannot be excluded. A reevaluation of the S-layer symmetry for *B. anthracis* now seems even more intriguing given the reported P2 symmetry for *B. thuringiensis* 4045 and the uncertain reports of P6 or P1 symmetry for *B. anthracis*.

Purifying EA1 in the absence of protease inhibitors led to the observation that the secreted EA1 was specifically processed to an 80-kDa form by an endogenous *B. anthracis* protease(s). This processing was duplicated in vitro with purified EA1 with proteases of different selectivity, suggesting the existence of a 15-kDa protease-sensitive loop or domain rather than specific proteolysis at defined amino acid residues. Similar specific proteolytic processing has been observed for the S-layer proteins of one other *Bacillus* species, *B. sphaericus* (12), and for two gram-negative organisms, *Campylobacter fetus* (5) and *Aeromonas salmonicida* (21). Removal of the protease-sensitive domain from the S-layer protein of *B. sphaericus* resulted in the inability to adsorb to cell walls but did not destroy the capacity for macromolecular assembly in vitro (12). The existence of domains with differential proteolysis sensitivities in EA1 lends further support to the concept of two domains, with the characteristics of each attributed to the folding characteristics and function of the domain (5). The functions and localizations of these proteolysis sensitive and -resistant domains of EA1 relative to the cell surface and within the S layer remain to be determined.

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